Mutagenic and genotoxic potential of pure Cylindrospermopsin by a battery of *in vitro* tests

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Highlights (for review)

- The *in vitro* mutagenicity and genotoxicity of Cylindrospermopsin (CYN) have been evaluated

- The Ames test, micronucleus (MN), mouse lymphoma and standard and modified comet assays were performed to evaluate CYN

- Only the MN assay with metabolic fraction S9 provided positive results suggesting pro-genotoxic potential for CYN

- Oxidative stress seems to be no related with CYN genotoxicity at tested conditions

1. Introduction

Cylindrospermopsin (CYN) is a cyanobacterial toxin considered as an emerging threat worldwide due to the progressive distribution of its main producer, *Cylindrospermopsis raciborskii* (Kinnear, 2010; Poniedzialek et al., 2012). CYN molecule consists of a tricyclic guanidine group combined with a hidroximethyl uracil (Ohtani et al., 1992). Its zwitterionic nature makes it highly water soluble and stable at extreme temperatures and pH (Falconer et al., 2006). Humans are more susceptible to the exposure to CYN in comparison to other cyanotoxins because up to 90% of total CYN is found outside the cyanobacterial cells (Rücker et al., 2007). In this sense, CYN has been documented as being involved in at least two epidemical cases of human poisoning (Carmichael et al., 2001; Griffiths and Saker, 2003) and it has been identified in some water reservoirs supplying drinking water (Bittencourt-Oliveira et al., 2014; Lei et al., 2014). Moreover, human exposure to CYN is possible through other different pathways such as bathing, recreational water activities, and, mainly, by the consumption of contaminated food (fish, mollusks, vegetables, etc.) (Gutiérrez-Praena et al., 2013). In fact, the bioaccumulation of CYN on fish (up to 2.7 ng/g, Messineo et al., 2010), crayfish (up to 4.3 mg/g, Saker and Eaglesham, 1999), mussels (up to 2.52 mg/g, Saker et al., 2004), lettuce (up to 8.029 µg/kg, Cordeiro-Araújo et al., 2017) etc. has been reported. In this regard, the European Food Safety Authority (EFSA) has recommended to collect more data about the toxicological profile of cyanotoxins, including CYN (Testai et al., 2016), and Humpage and Falconer (2003) proposed a tolerable daily intake of 0.03 mg CYN/kg of body weight.

The main target of CYN activity is the liver (Bernard et al., 2003; Zegura et al., 2011a). However, it has been also characterized as cytotoxic due to its negative effects on different organs (Runnegar et al., 1994; Terao et al., 1994; Falconer and Humpage, 2006; Guzmán-Guillén et al., 2013). One of CYN best-known modes of action is the irreversible inhibition of

protein synthesis (Terao et al., 1994; Runnegar et al., 1995; Froscio et al., 2003;) that leads to cytotoxic effects in a variety of cell cultures (hepatocellular, gastrointestinal tract and kidney cell lines) (Humpage et al., 2005; Froscio et al., 2009; Gutiérrez-Praena et al., 2012a). Moreover, some authors suggest that CYN cytotoxicity is also dependent on a cytochrome p-450 metabolism (Runnegar et al., 1995; Froscio et al., 2003) and the activation of CYN seems to enhance even more its toxicity (Norris et al., 2002; Humpage et al., 2005; Straser et al. 2011, 2013a). It has been shown that CYN induces oxidative stress mediated by reactive oxygen species (ROS) in a variety of cell types including mouse hepatocytes (Humpage et al., 2005; López-Alonso et al., 2013); fish leucocytes (Sieroskawska and Rymuszka, 2013), fish hepatocytes (Liebel et al., 2011) and cell lines from different origin such as fish liver (PLHC-1) (Gutierrez-Praena et al., 2011a); human colon (Caco-2) (Gutierrez-Praena et al., 2012a), umbilical vein endothelium (HUVEC) (Gutierrez-Praena et al., 2012b) and human hepatoma (Hep-G2) (Straser et al., 2013b).

On the other hand, different authors considered CYN a genotoxic compound due to its effects on DNA (Zegura et al., 2011a; Moreira et al., 2012). Also, some structural properties, such as the presence of uracil (Shen et al., 2002) or potentially reactive guanidine and sulfate groups (Bain et al., 2007) have led researchers to suggest a possible interaction of CYN with nucleic acids. Two mechanisms have been suggested for its genotoxic activity: 1) loss of kinetochore/spindle functions; 2) induction of DNA strand breaks (SBs) at DNA level (Sieroslawska, 2010). However, these mechanisms potentially involved in its genotoxicity are still under investigation.

In the scientific literature there are different reports dealing with the *in vitro* mutagenic/genotoxic effects of CYN using different assays (for a review see Pichardo et al., 2017). Thus, with respect to the Micronucleus (MN) assay, one of the preferred methods for assessing chromosome damage (EFSA, 2011), positive results have been obtained in different

experimental models. For example, Humpage et al. (2000) observed that CYN (6 and 10 μg/mL) increased the incidence of MN in the WIL2-NS lymphoblastic cell line after 48h exposure. Similar results were obtained by Straser et al. (2011) in Hep-G2 cells exposed to 0.05 and 0.5 μg/mL CYN and in peripheral blood lymphocytes (HPBLs) exposed to 0.1 and 0.5 μg/mL CYN as shown by Zegura et al. (2011b). In the common carp (*Cyprinus carpio* L.) leucocyte cell line 0.1 and 0.5 μg/mL CYN did not show genotoxic effects by the MN assay, but at 1 μg/mL, the frequency of MN was significantly higher (Sieroslawska and Rymuszka, 2015).

According to the studies performed with the comet assay, no DNA damage was detected on chinese hamster ovary (CHO-K1) cells after 24 h of treatment with CYN concentrations of 0.5 and 1 μg/mL (Fessard and Bernard, 2003) and on fish hepatocytes exposed to 0.1-10 μg/mL for 72h (Liebel et al., 2011). However, significant increases of DNA SBs were observed in mouse hepatocytes (0.05-0.5 μg/mL) after 18h exposure (Humpage et al., 2005), in HPBLs (0.05-0.5 μg/mL) after 4 h (Zegura et al., 2011b) as well as in Hep-G2 cells (0.01-0.5 μg/mL) after 12, 24 and 72 h (Straser et al., 2011; Straser et al., 2013b,c). Taking into account all these reports, it seems that the concentrations of CYN employed, the experimental model, and the type of assay used have a role on the results obtained. Moreover, there are additional data gaps regarding the genotoxic potential of CYN. Thus, although it is well known that CYN induces oxidative stress it has been not yet elucidated whether this mechanism contributes to CYN genotoxicity.

Studies concerning the influence of CYN on oxidative DNA damage *in vitro* are very scarce. Humpage et al. (2005) and Straser et al. (2013b) concluded that ROS is not a mediator of CYN genotoxicity. However, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG), a DNA oxidation product, was detected in a fish leucocyte cell line after 24 h treatment with CYN (Sieroslawska and Rymuszka, 2015). *In vivo*, DNA oxidation was also detected in tilapia sub-chronically exposed to environmental relevant concentrations (Guzmán-Guillén et al., 2013).

Another controversial point not yet fully elucidated is whether CYN is a genotoxic or a pro-genotoxic compound due to CYP-mediated metabolites (Zegura et al., 2011a; Straser et al., 2013a).

All these contradictory results and uncertainties increase the concerns about CYN genotoxicity and make necessary to elucidate its genotoxic potential. In this sense, the scientific opinion of EFSA on genotoxicity testing strategies applicable to food and feed safety assessment recommends a step-wise approach beginning with a basic battery of *in vitro* tests, comprising 1) a bacterial reverse mutation test (OECD 471), which detects gene mutations and 2) an *in vitro* mammalian cell micronucleus test (OECD 487), that covers both structural and numerical chromosome aberrations (EFSA, 2011). Regarding to the Ames test, there is only one report which evaluated the capacity of CYN as mutagenic agent, showing negative results (Sieroslawska, 2013) whereas the MN assay has shown positive results (Bazin et al., 2010; Straser et al., 2011; Hercog et al., 2017).

Taking into account these facts, the aim of this study was to investigate the potential mutagenicity and genotoxicity of CYN using a battery of different *in vitro* assays including: the bacterial reverse-mutation assay in *Salmonella typhimurium* (Ames test, OECD 471), the MN test (OECD 487), the standard comet assay on Caco-2 cells and the mouse lymphoma thymidine-kinase assay (MLA) on L5178Y*Tk*$^{+/-}$ cells, this one applied for the first time to study CYN genotoxicity. Moreover, to investigate the involvement of oxidative stress on CYN induced DNA damage, an enzyme-modified version of the comet assay was performed. In addition, the role of metabolism in CYN genotoxicity was studied by using the S9 fraction as metabolic activation system in the Ames, MN and mouse lymphoma assays in order to establish if CYN is a genotoxic or a pro-genotoxic cyanotoxin.

2. Materials and Methods

2.1. Supplies and chemicals

Cylindrospermopsin standard (purity 95%) was supplied by Alexis Corporation (Lausen, Switzerland). All assay chemicals were purchased from Sigma–Aldrich (Madrid, Spain), Gibco (Biomol, Sevilla, Spain), Moltox (Trinova, Biochem, Germany) and C-Viral S.L. (Sevilla, Spain).

2.2. Cells and culture conditions

For the Ames test, five *Salmonella typhimurium* histidine-auxotrophic strains TA97A, TA98, TA100, TA102 and TA104 were used. For the MN and MLA tests, L5178Y $Tk^{+/-}$ mouse lymphoma cells were originally provided by Dr. Olivier Gillardeux (Safoni-Synthélabo, Paris, France). Caco-2 cells, used for standard and enzyme-modified comet assays are derived from a human colon carcinoma (ATCC© HTB-37). L5178Y$Tk^{+/-}$ cells and Caco-2 cell line were cultured and maintained according to Mellado-García et al. (2017).

2.3. Test solutions

A stock solution of CYN (1000 µg/mL) was prepared in milliQ sterile water and maintained at less than 4°C. The exposure concentration solutions were made by dilution in sterile MilliQ water (Ames test), RPMI 1640 medium (MN and MLA assays) or MEM medium (standard and modified-comet assays).

2.4. Ames test

The mutagenicity test was performed following the principles of OECD guideline 471 (1997) with the modifications described in Mellado-Garcia et al. (2015), as follows. Briefly, cultures of five *Salmonella typhimurium* histidine-auxotrophic strains (TA97A, TA98, TA100, TA102 and TA104) were prepared from their main strain plates and used in their late

exponential growth phase. CYN mutagenic activity was assessed in three independent experiments using three technical replicates per concentration and per experiment, and in the absence and presence of S9 fraction as metabolic activation system from rat livers (S9 fraction induced by Aroclor-1254). The S9 fraction is an exogenous metabolising system that should be used when employing cells with inadequate endogenous metabolic capacity (OECD guideline 487). Each experiment included five increasing concentrations of CYN (0.625-10 µg/mL) selected according to Sieroslawska (2013). Higher CYN concentrations were not tested as they were not considered relevant. Also, a negative control (distilled sterile water) and a positive control (depending of the strains and presence or absence of S9 fraction) were included. Without S9 fraction for TA 97A/TA98/TA102/TA104 positive control was 2-nitrofluorene (2-NF) (0.1 µg/plate), and for TA100, sodium azide (NaN_3) (1 µg/plate). Positive control for all strains with S9 fraction was 2-aminofluorene (2-AF) (20 µg/plate). Results are expressed as revertant colonies and mutagenicity indexes (MI). The MI were calculated as the mean of revertant colonies for each concentration and strain divided by the mean of revertant colonies of the negative control (Mahon et al., 1989).

2.5. Micronucleus test

This assay was performed according to the OECD guideline 487 (2016) with the modifications described by Maisanaba et al. (2015). L5178Y*Tk*$^{+/-}$ cells were seeded at a concentration of 2.0×10^5 cell/mL and treated with five different concentrations of CYN (0-1.35 µg/mL in the absence of S9 fraction for 24h, and 0-2 µg/mL in the presence of S9 fraction for 4h). These concentrations were selected based on the mean effective concentrations (EC_{50}) obtained in a preliminary cytotoxicity test according to OECD 487 (2016) procedure. RPMI medium was used as a negative control, and 0.0625 µg/mL mitomycin (in absence of S9 fraction) or 8 µg/mL cyclophosphamide (in presence of S9 fraction) were used as positive

controls. After specific times of exposure (4 or 24h), cells were exposed to cythochalasin B (6 µg/mL) for 20 h. Then, cultures were centrifuged and the pellet was subjected to a hypotonic treatment with KCl. Afterwards the cells were again centrifuged and fixed. The resultant pellets were resuspended, dropped on microscope slides and stained with giemsa 10%. Micronuclei frequencies were scored in at least 2000 binucleated cells per concentration. The frequency of binucleated cells with micronuclei (BNMN) and the cytokinesis-block proliferation index (CBPI) were analyzed according to the recommendations of OECD 487 (2016).

2.6. Mouse lymphoma thymidine-kinase assay (MLA)

The MLA was performed according to the recommendations of OECD guideline 490 (2016) and Maisanaba et al. (2015). The experiment comprised a negative control (fresh medium), a positive control (methylmethanesulfonate, MMS 10 µg/mL without S9 fraction and cyclophosphamide, CP 3 µg/mL with S9 fraction), and six concentrations of CYN in the range 0-0.675 µg/mL (for 4 and 24h-assays). This range is based on a preliminary toxicity test conducted to determine the cytotoxicity of CYN by the relative total growth (RTG) after a treatment for 4h with and without S9 fraction and 24 h without S9 fraction. RTG values were used to decide on the acceptability of the toxicity at each concentration level, being the highest concentration selected for the mutagenic assay higher than 10-20% of RTG based on the recommendations of the ICH Expert Working Group (2008) and the OECD guideline. To evaluate the viability and mutagenic potential, cells were seeded at a density of 10^4 cells/mL in 96-well plates (two replicates per experimental group in each case) and, specifically for the mutation analysis, the replicates were also exposed to trifluorothymidine (TFT) at a final concentration of 4 µg/mL. After incubation for 12 days at 37 ºC and 5% CO_2 viable colonies and TFT mutation colonies were counted. In order to assist the scoring of mutant colonies, thiazolyl blue tetrazolium (MTT) (2.5 mg/mL) was added to each well and the plates were incubated for

4 h before counting. Colony size was estimated in a similar manner to that described by Honma et al. (1999); small (less than 1/3 of well diameter) or large (higher than 1/3 of well diameter). The induced mutant frequency (IMF) was determined according to the formula IMF = MF-SMF, where MF is the test culture mutant frequency and SMF is the spontaneous mutant frequency. Positive responses are determined as those that for any treatment meet or exceed the global evaluation factor (GEF, 126 x 10^{-6} for the microwell assay) and also when a positive trend is obtained.

2.7. Standard and enzyme-modified comet assay

The standard comet assay (single cell gel electrophoresis) is the most common method for measuring DNA damage in eukaryotic cells (Azqueta and Collins, 2013). Moreover, it has been modified to detect oxidized bases, by using the enzymes Endonuclease III (EndoIII), which recognizes damaged pyrimidines from double-stranded DNA, and Formamidopyrimidine DNA glycosylase (FPG), which detect oxidized purines including 8-oxo-guanine, generating apurinic/apyrimidinic-sites (AP-sites).

The genotoxicity of CYN was evaluated using the standard and modified comet assays, as previously described by Collins and Azqueta (2012) and Llana-Ruiz-Cabello et al. (2014). Caco-2 cells were plated at density of 3.5× 10^5 cells/mL into 24-well tissue culture plates and exposed for 24h or 48h to 0, 0.625, 1.25 and 2.5 µg/mL CYN. These concentrations were selected according to a cytotoxicity study previously performed by Gutierrez-Praena et al. (2012a). The most sensitive endpoint observed was the tetrazolium salt reduction (MTS); therefore, the EC_{50} value obtained for this endpoint at 24 h, 2.5 mg/mL CYN, was chosen as the higher exposure concentration, along with the fractions EC50/2 and EC50/4. After the exposure, Caco-2 cells were washed, trypsinized and resuspended in phosphate buffer saline (PBS) at 2.5 x 10^5 cell/mL. Briefly, the cell suspensions were mixed with pre-warmed (37^{O}C) 1%

(w/v) low-melting-point agarose in PBS, and immediately twelve aliquots of each cell suspension were placed on agarose precoated glass slides (one sample from each concentration was analyzed on each slide). Then, the slides were incubated in ice-cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4°C for at least 1 h. Afterwards, they were washed with enzyme buffer (buffer F) and two gels in each slide were exposed to lysis solution, enzyme buffer alone, buffer F containing FPG and enzyme buffer containing Endo III for up to 30 min in a metal-box at 37ºC. Finally, denaturing, electrophoresis, neutralizing, fixation, dying and quantification of the nuclei were carried out according to Mellado-García et al. (2016). The standard and modified comet assays were carried out three times by time assayed.

A negative control (medium without foetal bovine serum) and positive controls were included to monitor the assays. Cells were treated with 100 µM of H_20_2 as positive control for the standard comet assay (DNA SBs) and Endo III-sensitive sites. The positive control for FPG-sensitive sites was 2 µM of Ro 19-8022 photosensitiser (Hoffman-La Roche, Switzerland) combined with light irradiation (1.5 min).

The results were expressed as mean % DNA in tail or tail intensity, which corresponds to the intensity of the comet tail relative to the total intensity (tail plus head) and reflects the amount of DNA breakage (Kumaravel et al., 2009). To calculate the net FPG or Endo III-sensitive sites, the median % DNA in tail in the slide treated with buffer F was subtracted from the % DNA in tail in slide treated with enzyme.

2.8. Statistical analysis

Statistical analysis was performed using the analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. All analyses were performed using Graph-Pad InStat

software (Graph-PadSoftware Inc., La Jolla, USA) and IBM SPSS Statistics (Madrid, Spain). Differences were considered significant at *p<0.05, **p<0.01 and ***p<0.001, respectively.

3. Results

3.1 Ames test

The results obtained after CYN exposure did not indicate significant differences in TA97A, TA98 and TA100 bacterial strains under lab conditions tested (Table 1). In the case of TA102 bacterial strain exposed to pure CYN it showed significant differences in absence of metabolic fraction (1.25-10 µg/mL) and only at 5 µg/mL in presence of S9 fraction. For TA104, statistical differences were only observed at 0.625 µg/mL (in absence and/or presence of the external metabolic activation) and at 10 µg/mL (in absence of S9 fraction). However, it has to be pointed out that the statistical significance is not the only determining factor for a positive result (OCDE 471). The results are considered positive (mutagenic) when there is at least a 2-fold increase over the control (MI higher than 2) in at least one bacterial strain without or with metabolic activation (Mortelmans and Zeiger, 2000). In this case, for all strains the MI were less than 2. Consequently, the results proved that CYN was not mutagenic under the conditions tested. No signs of precipitation and/or toxicity were observed under the conditions studied. Positive controls produced statistically significant increases in the number of revertant colonies (p<0.01), as well as the MI was higher than 2, confirming the sensitivity of the test system and the activity of the S9 fraction.

3.2 Micronucleus test

CYN induced a significant increase in the frequency of BNMN in presence of S9 fraction at a concentration range between 0.25-2.0 µg/mL (p<0.05; p<0.01) compared to the control group (Table 2). However, no significant variations in BNMN % were observed at the lowest

concentration tested (0.13 µg CYN/mL) nor in absence of the metabolic fraction (in the range 0.08-1.35 µg CYN/mL) compared to their respective control groups. Regarding to the CBPI, no significant variations were observed in presence nor absence of S9 fraction at any concentration tested in comparison to the control group. Moreover, positive controls induced a significant increase (p<0.05; p<0.001) in the frequency of MN in presence and absence of S9 fraction.

3.3 Mouse lymphoma thymidine-kinase assay (MLA)

The mutagenicity results obtained in the MLA after exposure of L5178Y $Tk^{+/-}$ cells with CYN for 4 (with and without S9 fraction) and 24h are shown in Table 3. CYN did not induce a mutagenic response at any concentration tested after both times of exposure. Moreover, the use of S9 fraction did not influence the results. Positive controls (10 µg/mL MMS without S9 fraction and 3 µg/mL CP with S9 fraction) were run in parallel, showing significantly increased mutant frequencies. Both, negative and positive controls fulfil the acceptance criteria of the OECD 490 guideline. Also, a good concordance was observed between the two conducted experiments for each exposure time.

3.4 Standard and enzyme-modified comet assays

In the standard comet assay, CYN (0-2.5 µg/mL) did not induce DNA breaks in Caco-2 cells at any concentrations tested after 24 and 48 h of exposure (Fig. 1a). For cells treated with 100 µM of H_20_2, a significant (p<0.01) increase in DNA breaks was observed after both times of exposure.

CYN did not induce oxidation of pyrimidines and purines as revealed the results obtained after application of Endo III or FPG enzymes, respectively (Fig. 1b). Both positive

controls (H_20_2 and Ro-19-8022 plus 5 min of 500 W visible light) showed significant increases of % tail DNA (p<0.01) under the conditions tested.

4. Discussion

The fact that EFSA requires data on the toxicity profile of cyanotoxins for their correct risk assessment (Testai et al., 2016), makes the study of CYN genotoxicity a topic worth of research. In this sense, a thorough genotoxicity evaluation of CYN by *in vitro* assays has been performed including the Ames test, MN, MLA and the comet assays.

With regard to the Ames test, the present study shows that CYN was non-mutagenic with and without S9 fraction using five *S. typhimurium* strains (TA97A, TA98, TA100, TA102 and TA104). Among the strains used, TA102 and TA104 were the only responsive ones although the MI were ≤2. This could be related to the already known CYN capacity to induce oxidative stress, because as stated by Mortelsman and Zeiger (2000), strains TA102 and TA104 may be used if it is suspected that the chemical may induce oxidative damage, or be a DNA cross-linking agent. TA102, the most responsive strain in this case, is especially sensitive for detecting DNA cross-linking damage because it has an intact (wild-type) DNA excision repair mechanism which is required for the repair of that kind of damage.

Similarly to our results, Sieroslawska (2013) reported that CYN exhibit no mutagenic activity in the Ames microplate format mutagenicity assay for the strains used, *S. typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 uvrA and WP2 [pKM101] with or without metabolic activation with S9 fraction. In the case of CYN, no more data are available so far about its mutagenic potential by this test.

No single mutagenicity test can detect all types of potential human mutations with 100% accuracy or prediction (Pathak et al., 2017). Moreover, Kirkland et al. (2011) concluded

that data from Ames and *in vitro* MN tests allow the detection of all relevant toxicants causing *in vivo* carcinogenicity. In the present work, the MN test results indicated no significant variations in the frequency of MN in binucleated cells in absence of exogenous metabolic activation S9 fraction in comparison to the negative control group. However, the increment of BNMN frequency observed in the presence of S9 fraction indicated that potential genotoxic damage could be due to its metabolites. It has been reported that metabolic transformation of CYN is crucial for toxin-induced toxicity, and particularly for its genotoxicity (Fessard and Bernard, 2003; Bain et al., 2008). Similarly to our results, no significant difference was observed in the frequency of MN in the undifferentiated human hepatoma cell line HepaRG exposed to CYN (Bazin et al., 2010). However, in differentiated HepaRG cells (with CYN-metabolizing capability as indicated by the authors) the frequencies of BNMN increased from 0.04 µg/mL to 0.3 µg/mL CYN. This different genotoxic response between undifferentiated and differentiated HepaRG evidenced that metabolism of CYN was required. The same authors observed that there was a significant increase in the frequency of BNMN in undifferentiated and differentiated Caco-2 cells (Bazin et al., 2010). There are other authors that have also reported CYN genotoxicity by the MN assay. Thus, Humpage et al. (2000) demonstrated that CYN produced cytogenetic damage *in vitro* as it clearly induced whole chromosome and chromatics loss in the range 1-10 µg/mL after 24h exposure in human WIL2-NS lymphoblastoid cell line. Similarly, CYN induced MN in HepG2 cells after 24 h of exposure (Straser et al., 2011; Hercog et al., 2017) and in HPBLs after 4 and 24 h (Zegura et al., 2011b). Moreover, CYN induced genotoxic effects in fish CLC cell line using the MN assay with the highest toxin concentration used (1µg/mL) (Sieroslawka and Rymuszka, 2015). Nevertheless, no data on CYN metabolite identification were available in WIL2-NS, HepG2, HPBLs and CLC cell lines.

Nowadays, the process underlying the metabolic activation of CYN is not yet elucidated (Kittler et al., 2014). In the present work, S9 fraction was used, which is a co-factor-

supplemented post-mitochondrial fraction prepared from the livers of rodents treated with a combination of phenobarbital and β-naphthoflavone, according to OECD 487. These enzymatic inductors increase the levels of several CYP enzymes, in particular the CYP1A subfamily of enzymes (CYP1A1 and CYP1A2) (Ku et al., 2007). Our results indicated that this exogenous metabolising system, which induces mixed-function oxidases, is involved in the genotoxic effects of CYN in L5178Y T$k^{+/-}$ cells. In this sense, Straser et al. (2011) and Hercog et al. (2017) showed that exposure of HepG2 cells to CYN induced the up-regulation of CYP genes *CYP1A1* and *CYP1A2* providing further evidence that these enzymes are involved in the metabolic activation of CYN. Similar explanations were also proposed by Humpage et al. (2005) who observed that CYP450-derived metabolites were responsible for genotoxicity induced by CYN-exposed hepatocytes.

Apart from the *in vitro* mammalian cell MN test, there is also a different assay commonly used to investigate chromosome aberrations, the *in vitro* mammalian chromosomal aberration test (EFSA, 2011). Lankoff et al. (2007) observed no significant influence on the frequency of asymmetrical structural chromosome aberrations in CHO-K1 cells exposed to CYN with and without S9 fraction after performing this test.

In the present study, there is a lack of agreement between the negative results obtained in bacterial cells (Ames test) in presence of external metabolic activation and the positive results obtained in the *in vitro* mammalian cell MN test with S9 fraction. This could be explained by differences in the concentrations used, the different experimental models and the duration of the exposure in each assay (Kirkland et al., 2014). In any case, both assays measure different endpoints, therefore their results are not contradictory but complementary.

Based on our findings and following the criteria of EFSA (2011), as the Ames test and the MN assay have shown different results, it may be appropriate to conduct further testing *in vitro*. In this regard, two additional genotoxicity tests have been performed, the MLA and the comet assay. In relation to the MLA, this is the first time that it has been applied to study CYN genotoxicity. This test has the potential to detect mutagenic events at the thymidine kinase (*tk*) locus of L5178Y mouse lymphoma *tk* (+/-) cells by measuring resistance to the lethal nucleoside analogue TFT (Lloyd and Kidd, 2012). This test, along with the Ames test, is considered by EFSA (2011) one of the most commonly used *in vitro* methods to investigate gene (point) mutations. Moreover, this test can detect also other genetic events leading to the inactivation or loss of heterozygosity of the *tk* gene, such as large deletions or mitotic recombination (EFSA, 2011). The negative results obtained with absence and presence of S9 fraction agree with those of the another mutagenicity test performed, the Ames test.

The standard comet assay was performed on Caco-2 cells, as human intestine is a target tissue for xenobiotics after oral exposure. It is based on the ability of a SB to relax supercoiling in a loop of DNA, thus allowing the DNA to extend to the anode during electrophoresis forming a comet-like image in which the relative intensity of the comet tail reflects the break frequency (Azqueta and Collins, 2016). This assay is the most frequently used to evaluate the genotoxicity of CYN. In this work, CYN did not induce DNA SBs after 24 or 48 h at any of the assayed concentrations in Caco-2 cells. Similar results were obtained by Fessard and Bernard (2003) in CHO-K1 cells exposed to CYN (0.5 and 1 µg/mL) for 24h, by Liebel et al. (2011) in *Prochilodus lineatus* hepatocytes exposed for 72 h to 0.1, 1 and 10 µg/mL, and by Silva et al. (2017) in *Hoplias malabaricus* hepatocytes exposed for 72h to 0.1-100 µg/L. Also, CYN after 4 h exposure did not induce % DNA in tail in the metabolically competent human hepatoma cell line HepG2 (Straser et al., 2011; 2013b). Nevertheless, after

prolonged exposure (12, 24, 18 and 72 h), CYN (0.01- 0.5 µg/mL) induced formation of DNA double SBs in this cell line (Straser et al., 2011; 2013b,c). This was also confirmed by Hercog et al. (2017) in the same cellular type as no SBs were observed at 4h, but positive results were obtained after 24h CYN exposure in a similar concentration range. Moreover, additional reports evidencing the genotoxicity of CYN by the comet assay are available in the scientific literature. Thus, a significant concentration-dependent increase in comet tail moment of nuclei from mouse primary hepatocytes treated with 0.05 to 0.5 µM CYN for 18 h was observed by Humpage et al. (2005). Similarly, carp leukocytes exposed to 0.5 µg/mL CYN for 18 h showed a high level of DNA damage (Sieroslawska and Rymuszka, 2013). Zegura et al. (2011b) showed that in HPBLs non-toxic concentrations of CYN induced a significant increase of DNA SBs after 4 and 24 h of exposure. One more time, the variability in experimental models, exposure times and concentrations used could explain the differences observed.

As it has been shown above, CYN genotoxicity by the comet assay has been widely investigated. However, the modified version of the comet assay in order to elucidate the potential contribution of oxidative stress on CYN genotoxicity has been scarcely applied. This is noteworthy taking into account that it is well documented that CYN produces changes in some oxidative stress biomarkers such as ROS content *in vitro* (Humpage et al., 2005; Liebel et al., 2011; Gutierrez-Praena et al., 2011a; 2012a,b; López-Alonso et al., 2013; Straser et al., 2013b; Sieroskawska and Rymuszka, 2015). Oxidative stress and ROS formation could be the cause of CYN genotoxicity. The importance of DNA oxidations is emphasized by their mutagenic potential, although they have multiple additional roles in aging and cancer, including, e.g., mitochondrial function, microsatellite instability and telomere shortening (Evans et al., 2004). The use of lesion-specific enzymes has added a higher value to the standard comet assay, and FPG in particular has been used in many investigations of oxidative damage to DNA (Azqueta

and Collins, 2013). In the present study, the alkaline comet assay in combination with Endo III and FPG were used to detect oxidized pyrimidines and purine bases, respectively (Collins and Azqueta, 2012). The enzymes induce additional breaks at the sites of oxidized bases and increase the DNA in the tail of the comets (Glei et al., 2016). Our results show that CYN did not show DNA-oxidative effects when Endo III and FPG were used. With this assay, it was demonstrated that ROS are not mediators of CYN genotoxicity at the experimental conditions employed. There is only an additional study by Straser et al. (2013b) that applied the modified version of the comet assay to investigate the genotoxicity of up to 0.5 µg/mL CYN in HepG2 cells, but only FPG was used. The authors concluded that the DNA damage caused by CYN was unlikely to be due to oxidative stress, although increased ROS levels were detected. This observation was in accordance with the results obtained *in vitro* by Humpage et al. (2005) as they observed no changes in malondialdehyde (a biomarker of lipid peroxidation) in CYN-exposed mouse hepatocytes. *In vivo*, in liver of fish, DNA oxidation was not evidenced (Gutiérrez-Praena et al., 2011b). These negative results could be explained as some DNA oxidation products are repaired by enzymes of the base excision repair (BER) pathway (David et al., 2007; Loft and Moller, 2006) maintaining the DNA its original structure. However, other works reported that there is a direct connection between CYN-mediated induction of oxidative stress and DNA damage, such as Sieroslawska and Rymuszka (2015) in a carp leucocyte cell line after 24 h treatment, or Guzmán-Guillén et al. (2013) in liver and kidney of tilapia fish exposed to 100 µg CYN/L for 7 or 14 days.

To sum up, regarding the four different genotoxicity/mutagenicity assays performed, only positive results were obtained in the MN test with the metabolic fraction S9. This implies that CYN metabolites can induce chromosomal damage. In this regard, it has been suggested that the parent compound and the possibly formed metabolites could exert toxicity with a

different mechanism, also depending on CYN concentrations (Buratti et al., 2017). Unfortunately, CYN metabolites have not been identified so far. The discrepancies observed among our results and those reported by the scientific literature could be due to different reasons such as 1) the variety of experimental models, 2) differences in uptake; 3) the range of concentrations assayed; 4) differences in the metabolic status of the cell lines (Huguet et al., 2014) including CYP expression and presence of tissue-specific CYP isoforms (Bazin et al., 2010); 5) exposure times; 6) DNA repair processes and 7) the use of no standard assay protocols.

Overall, our results suggest that CYN could be a pro-genotoxic compound in mammalian *in vitro* models as positive results were only obtained when the metabolic fraction S9 was used in L5178Y $Tk^{+/-}$ cells. Moreover, oxidative stress seems to be not directly related to genotoxicity as the modified version of the comet assay provided negative results at the conditions tested. These data will contribute to a more realistic risk assessment of this prevalent cyanotoxin in waters and food.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

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Tables and figure captions:

Table 1. Results of the Ames test conducted with CYN. Milli Q water was used as negative control and DMSO (10 µL) as solvent for positive controls. Data are given as mean ± SD revertants/plate of 3 independent experiments and mutagenicity index (MI). Positive controls without S9 fraction for TA 97A/TA98/TA102/TA104: 2-Nitrofluorene (0.1µg/plate) and for TA100: NaN_3 (1 µg/plate). Positive control for all strains with S9 fraction: 2-Aminofluorene (20 µg/plate). ** p<0.01 significantly different from negative controls.

Table 2. Percentage of binucleated cells with micronuclei (BNMN) and cytokinesis-block proliferation index (CBPI) in cultured mouse lymphoma cells L5178Y*Tk* $^{+/-}$ exposed to CYN. The genotoxicity assay was performed in absence and presence of the metabolic fraction S9. The values are expressed as mean ± SD The significance levels observed are * p<0.05, ** p<0.01, *** p<0.001 in comparison to control group values (negative control= medium).

Table 3. Toxicity and mutagenicity of CYN in L5178Y*Tk* $^{+/-}$ cells after 4h without S9 fraction (a) 4h with S9 fraction (b) and 24h without S9 fraction (c) by the MLA. *** p<0.001 significantly different from negative control (medium). Positive controls: methylmethanesulfonate, MMS 10 µg/mL without S9 fraction and cyclophosphamide, CP 3 µg/mL with S9 fraction. [a] Total mutant frequency divided into small/large (S/L) colony mutant frequencies. The induced mutant frequency (IMF) was determined according to the formula IMF = MF-SMF, where MF is the test culture mutant frequency and SMF is the spontaneous mutant frequency.

Figure 1. Effect of CYN on DNA strand-breaks (SBs) and net Endo-FPG-sensitive sites of Caco-2 cells exposed for 24 and 48 h using the standard alkaline (a) and modified-comet assay (b). The levels of DNA SBs, oxidized pyrimidines and oxidized purines are expressed as % tail DNA. All

values are expressed as mean ± SD of three independent experiments. The significance levels observed are ** $p<0.01$ in comparison to negative control group values (medium). Positive controls: 100 µM H_2O_2 for the standard comet assay and Endo III-sensitive sites, and 2 µM of Ro 19-8022 photosensitiser with light irradiation for FPG-sensitive sites.

Table 1

Table 1

	µg/mL	TA 97A				TA98				TA100				TA102				TA104			
		-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI	-S9	MI	+S9	MI
Pure CYN	Negative controls	239±31	-	247±25	-	18±4	-	26±4	-	92±7	-	98±14	-	230±16	-	253±79	-	294±34	-	307±19	-
	0.625	246±17	1± 0.1	259±40	1± 0.2	19±2	1.1± 0.1	36±4	1.5± 0.1	132±18	1.5± 0.3	127±19	1.3± 0.2	219±16	1.0± 0.1	293±25	1.2± 0.1	402±62**	1.5± 0.1	457±5**	1.5± 0.0
	1.25	194±23	0.7± 0.1	216±28	0.9± 0.1	20±2	1.1± 0.1	30±1	1.2± 0.2	126±24	1.3± 0.3	125±21	1.3± 0.2	322±20**	1.4± 0.1	239±36	0.9± 0.1	314±20	1.1± 0.1	316±50	1± 0.2
	2.5	198±9	0.8± 0.0	239±45	1± 0.2	23±3	1.3± 0.1	32±4	1.3± 0.1	123±7	1.2± 0.0	114±5	1.3± 0.2	294±10**	1.3± 0.0	329±36	1.3± 0.1	332±43	1.2± 0.2	360±43	1.2± 0.1
	5	251±48	1± 0.3	339±27	1.4± 0.1	17±2	0.9± 0.1	36±2	1.2± 0.2	117±8	1.3± 0.0	108±13	1.2± 0.0	342±31**	1.5± 0.2	409±5**	1.6± 0.0	323±20	1.1± 0.1	306±51	1± 0.2
	10	198±16	0.8± 0.0	239±11	1± 0.0	21±0	1.1± 0.0	38±8	1.4± 0.3	97±5	1.3± 0.1	98±9	1.2± 0.1	309±12**	1.3±0.0	320±22	1.3± 0.1	216±26*	0.7± 0.0	257±13	0.8± 0.0
	Positive controls	627±54**	2.5± 0.2	547±42**	2.2± 0.2	1164±20**	63.2± 1.1	1011±75**	38.4± 2.9	324±3**	3.5± 0.1	370±14**	3.8± 0.1	617±16**	2.7±0.1	632±27**	2.5± 0.1	666±35**	2.3± 0.1	642±26**	2.1± 0.1
	DMSO	242±23	1± 0.1	273±34	1.1± 0.1	22±3	1.2± 0.2	21±3	0.8± 0.1	87±9	0.9± 0.1	86±8	0.9± 0.1	294±9	1.3±0.0	318±18	1.3± 0.1	282±32	1± 0.1	294±25	1± 0.1

Table 2

Table 2

Test substance	Absence of S9				Presence of S9			
	Exposure time (h)	Concentration (µg/mL)	BNMN (%) ± SD	CBPI ± SD	Exposure time (h)	Concentration (µg/mL)	BNMN (%) ± SD	CBPI ± SD
Negative control	24	-	0.5±0.1	1.9±0.1	4	-	0.4±0.0	1.9±0.0
Positive control	24	Mitomycin C 0.0625	7.6±1.1***	1.7±0.0	4	Cyclophosfamide 8	1.3±0.3*	1.8±0.0
CYN	24	0.08	0.4±0.2	1.8±0.0	4	0.13	0.8±0.3	1.8±0.1
	24	0.16	0.1±0.2	1.6±0.2	4	0.25	1.3±0.2*	1.8±0.0
	24	0.33	0.4±0.6	1.8±0.1	4	0.5	1.5±0.1**	1.8±0.1
	24	0.67	0.2±0.3	1.6±0.2	4	1.0	2.1±0.4**	1.9±0.1
	24	1.35	0.4±0.2	1.4±0.1**	4	2.0	2.0±0.9**	1.8±0.1

Table 3

Table 3a. In absence of S9 (4h)

Concentration (µg/mL)	Relative total growth		Percent plating efficiency		Mutant frequency (x 10^{-6})		MF (S/L)[a]		IMF (MF-SMF) (x 10^{-6})	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	100	100	120	113	69,1	94,3	41/28	61/34	-	-
0.042	95	103	143	116	118	146	96/22	119/27	49	52
0.084	102	110	135	100	102	137	82/20	110/27	33	43
0.168	86	109	127	111	113	130	97/16	111/19	44	36
0.338	92	108	113	102	146	162	119/27	132/30	77	68
0.675	75	82	124	67	127	170	91/36	121/49	58	76
MMS (10 µg/mL)	58	63	88	35	1880***	1080***	1198/682	688/392	1810	986

Table 3b. In presence of S9 (4h)

Concentration (µg/mL)	Relative total growth		Percent plating efficiency		Mutant frequency (x 10^{-6})		MF (S/L)[a]		IMF (MF-SMF) (x 10^{-6})	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	100	100	76	118	132	107	63/69	55/45	-	-
0.042	90	97	80	88	145	127	86/84	67/60	13	20
0.084	71	95	88	87	160	128	80/79	63/66	27	21
0.168	68	61	84	90	189	156	163/98	122/34	56	49
0.338	41	55	106	140	177	190	106/71	67/30	44	83
0.675	37	29	81	76	154	161	88/67	93/68	22	54
CP (3 µg/mL)	52	69	96	71	1020***	1555***	519/483	896/654	888	1448

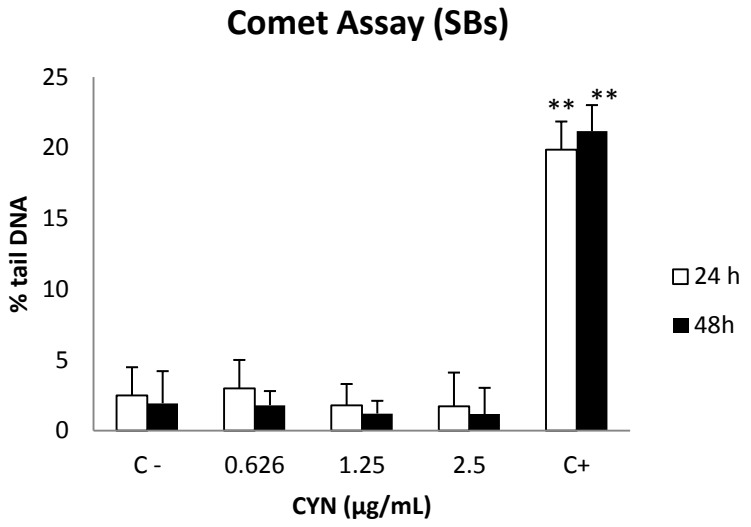
Table 3c. In absence of S9 (24h)

Concentration (µg/mL)	Relative total growth		Percent plating efficiency		Mutant frequency (x 10^{-6})		MF (S/L)[a]		IMF (MF-SMF) (x 10^{-6})	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	100	100	93	76	115	171	95/20	136/40	-	-
0.042	65	97	100	74	207	124	150/57	70/54	91	-48
0.084	62	96	111	67	194	154	128/66	90/64	79	-17
0.168	58	91	127	88	121	148	56/65	88/60	6	-24
0.338	64	61	113	56	162	181	93/69	129/135	47	9
0.675	30	26	58	65	141	231	78/63	180/51	26	59
MMS (10 µg/mL)	27	30	34	25	2410***	3220***	1672/738	1814/1106	2290	3050

Figure 1

Fig.1

a)



b)